PLANTS HAVING ENHANCED GALL RESISTANCE AND METHODS AND COMPOSITIONS FOR PRODUCING SAME

CROSS REFERENCE TO RELATED CASES

5   This application claims priority from a co-pending U.S. Provisional Application No. 60/107,185, filed November 5, 1998, which is incorporated herein by reference.

FIELD OF THE INVENTION

10   This invention is directed generally to compositions and methods for producing plants resistant to gall disease. The methods involve transforming plants with nucleic acid constructs encoding sense-strand untranslatable RNA molecules and/or double-stranded RNA molecules, RNA molecules that are similar to (but not identical to) certain *Agrobacterium* tumor-inducing genes.

15

BACKGROUND OF THE INVENTION

Several different recombinant DNA technologies have been employed to interfere with gene expression in plants including technologies based on ribozyme molecules, antisense RNA molecules, and co-suppression constructs. For a general
20   discussion of ribozyme technology, antisense technology and co-suppression technology, see Castanotto, *Crit. Rev. Eukaryot. Gen. Expr.* **2**:331-357, 1997; Simons, *Gene*, **72**:35-44, 1988; and Jorgensen, *Biotechnol.* **8**:340-344, 1990, respectively.

In addition to standard co-suppression techniques, researchers have studied
25   the suppression of a target gene caused by the introduction of an untranslatable sense-strand transgene. (Dougherty and Parks, *Curr. Opin. Cell Biol.* **7**:399-405, 1995; Jorgensen et al., *Science*, **279**:1486-1487, 1998). This method has been used to silence a number of endogenous plant genes, as well as viral genes. (Marano and Baulcombe, *Plant J.* **13**:537-546, 1998; Conner et al., *Plant J.* **11**:809-823, 1997,
30   Dougherty et al., *Mol. Plant-Microbe Interact.* **7**:544-552, 1994; and Smith et al., *Plant Cell* **6**:1441-1453, 1994).

The bacterial genus *Agrobacterium* contains several species that cause disease in economically important plants. For example, *Agrobacterium tumefaciens* causes gall tumors when it infects the wounded tissue of certain dicotyledonous plants such as apple trees and grape vines (DeCleene and DeLey, *Bot. Rev.* **42**:389-

5   466, 1976). Pathogenic strains of *Agrobacterium* harbor a tumor-inducing (Ti) plasmid that carries genes essential for gall tumorigenesis (Watson et al., *J. Bacteriol.* **123**:255-264, 1975; Van Larebeke et al., *Nature* **252**:169-170, 1974). The transferred DNA ("T-DNA"; **Figure 1**) portion of the Ti plasmid enters the cells of susceptible plants and integrates into nuclear DNA (Chilton et al., *Cell* **11**:263-

10   271, 1977; Willmitzer et al., *Nature* **287**:359-361, 1980; Chilton et al., *Proc. Natl. Acad. Sci. USA* **77**:4060-4064, 1980). Following T-DNA integration in cellular DNA, expression of three T-DNA genes, *iaaM, iaaH*, and *ipt,* leads to overproduction of the plant growth hormones auxin and cytokinin, resulting in tumorous growths referred to as "galls." (Ream, *Annual Rev. Phytopathol.* **27**:583-

15   618, 1989; Winans, *Micro. Revs.* **56**:12-31, 1992; and Zambryski, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**:465-490, 1992) (**Figure 2**). The *iaaM, iaaH*, and *ipt* genes have been well characterized, and the sequences of these genes are available for example in the Genbank database under accession numbers X56185, M25805, and X17428, respectively.

20   *iaaM* and *iaaH* are required for auxin production: the enzyme encoded by *iaaM* converts tryptophan into indole acetamide, which the enzyme encoded by *iaaH* converts into indole acetic acid, an auxin (Schroeder et al., *Eur. J. Biochem.* **138**:387-391, 1984; Thomashow et al., *Proc. Natl Acad. Sci.* **81**:5071-5075, 1985; Thomashow et al., *Science* **231**:616-618, 1986; and Inze et al., *Mol. Gen. Genet.*

25   **194**:265-274, 1984). Loss of either enzyme prevents auxin production. *ipt* is required for cytokinin production; the enzyme encoded by this gene converts adenosine monophosphate into isopentenyl adenosine monophosphate, a cytokinin (Akiyoshi et al. *Proc. Natl. Acad. Sci. USA* **81**:5994-5998, 1984; Barry et al., *Proc. Natl. Acad. Sci. USA* **81**:4776-4780, 1984; and Buchmann et al., *EMBO J.* **4**:853-

30   859, 1985). Inactivation of *ipt* and either one of the two auxin-biosynthesis genes on

the Ti plasmid will reduce gall formation (Ream et al., *Proc. Natl. Acad. Sci. USA*
80:1660-1664, 1983).

Additionally, galls can be formed in cases in which just the cytokinin
pathway, or only the auxin biosynthesis pathway, is functional. In these cases the
galls are visually distinguishable based on their different morphological
characteristics. For example, plants infected with an *Agrobacterium* strain that
carries only the gene necessary for the production of auxin develop necrotic galls. In
contrast, plants infected with strains of the bacteria that induce only the production
of cytokinin produce shooty galls.

To date, adequate means do not exist to control gall disease on grape vines,
fruit trees, nut trees, chrysanthemums, roses, cane berries, ornamental shrubs, and
other nursery crops. Inoculation of plants with *Agrobacterium radiobacter* strain
K84 affords some protection against specific strains of *A. tumefaciens* (Moore,
Microbiol. Sci. **5**:92-95, 1988); however, gall disease remains a multimillion dollar
worldwide problem. *Arabidopsis thaliana* plants resistant to gall disease have been
produced by a traditional genetic approach (Nam et al., *Plant Cell* **9**:317-333,
1997), but this strategy currently is not applicable to plants in which gall is a
problem.

This invention is directed towards a new, more effective method of
producing plants that are substantially resistant to gall disease caused by bacteria.

SUMMARY OF THE INVENTION

The present invention provides compositions that can be used to prevent
tumor (gall) formation in plants infected with bacterial pathogens such as
Agrobacterium tumefaciens and other species of *Agrobacterium*. These
compositions comprise nucleic acid molecules that, when introduced into plants, can
produce transgenic plants having enhanced resistance to tumors caused by such
pathogens. The invention also encompasses transgenic plants that contain these
nucleic acid molecules.

        In general terms, the invention involves the use of nucleic acid constructs
that encode untranslatable single-stranded RNA, double-stranded RNA, and/or
untranslatable double-stranded RNA molecules that share specified high levels of
sequence identity with target genes in the bacterial pathogen. For example, the
5   constructs may encode one or more types of untranslatable RNA molecules sharing
high levels of sequence identity with one or more of the *iaaM, iaaH,* or *ipt* tumor
genes of *Agrobacterium*. The invention also provides nucleic acid constructs that
encode multiple different untranslatable single-stranded RNA, double-stranded
RNA, and/or untranslatable double-stranded RNA molecules, including both sense
10   and antisense RNA molecules. For example, one construct provided by the
invention encodes untranslatable RNA forms of both the *ipt* and *iaaM* genes of
Agrobacterium tumefaciens; this construct can suppress both shooty and necrotic
gall formation in plants caused by a pathogenic strain of *A. tumefaciens* infecting the
plant.

15   The untranslatable RNA molecules appear to inhibit gall formation by
triggering sequence-specific destruction of RNA molecules encoded by certain
"target genes" of the infecting pathogen in the plant. However, the mechanism by
which this occurs is not fully understood. For optimal efficacy, the untranslatable
single-stranded RNA, double-stranded RNA, and/or untranslatable double-stranded
20   RNA molecules should be of a sufficient length and share a minimum degree of
sequence identity with the target bacterial gene such that disease resistance is
conferred to the host cell. The level of disease resistance conferred to the host cell
by the RNA molecule can be determined using the experiments described below.
Thus, the RNA molecules encoded by the constructs provided herein are typically at
25   least 25 nucleotides in length and share at least 60% sequence identity with the
target gene of the pathogen. However, enhanced tumor suppression may be obtained
by increasing the length of the untranslatable single-stranded RNA, double-stranded
RNA, and/or untranslatable double-stranded RNA molecules (e.g., to at least 100, or
at least 200 nucleotides) and/or by enhancing the level of sequence identity with the
30   target gene (e.g., to at least 70%, 75%, 80%, or 90% sequence identity).

The invention also provides transgenic plants containing one or more of the above-described nucleic acid constructs. These constructs may be introduced into any plant species, but commercial crop plants for which *Agrobacterium* is a significant pathogen are expected to be particularly benefited by this technology.

5   The invention also provides chimeric plants that include both untransformed cells and transformed cells. These plants display "conferred resistance" in that the transgenic portion confers disease resistance to the non-transgenic portion. The chimeric plants are particularly useful for generating resistant, but not transgenic, fruits and other consumable portions of plants that are not transgenic.

10

SEQUENCE LISTINGS

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three-letter code for amino acids. Only one strand of each nucleic acid sequence is shown,

15   but the complementary strand is understood as included by any reference to the displayed strand.

SEQ ID NO: 1 is a primer used to produce the mutant *iaaM* gene.

SEQ ID NO: 2 is a primer used to produce the mutant *iaaM* gene.

SEQ ID NO: 3 is a primer used to produce the mutant *ipt* gene.

20   SEQ ID NO: 4 is a primer used to produce the mutant *ipt* gene.

SEQ ID NO: 5 is the DNA sequence that encodes an untranslatable RNA molecule having a high sequence identity withthe *Agrobacterium ipt* gene.

SEQ ID NO: 6 is an amino acid sequence that is encoded by the untranslatable RNA molecule depicted in SEQ ID NO: 5.

25   SEQ ID NO: 7 is a DNA sequence encoding the untranslatable RNA molecule having a high sequence identity with the *Agrobacterium iaaM* gene.

SEQ ID NO: 8 is an amino acid sequence encoded by the untranslatable RNA molecule depicted in SEQ ID NO: 7.

SEQ ID NO: 9 is the nucleic acid sequence of the *ipt/iaaM* untranslatable

30   construct.

SEQ ID NO: 10 is the nucleic acid sequence of the *iaaM* gene.

SEQ ID NO: 11 is the nucleic acid sequence of the *iaaH* gene.

SEQ ID NO: 12 is the nucleic acid sequence of the *ipt* gene.

5   **DESCRIPTION OF DRAWINGS**

Figure 1 is a diagram of the *A. tumefaciens* transferred DNA ("T-DNA") that contains three oncogenes: *iaaM, iaaH,* and *ipt*. Triangles represent T-DNA border sequences which delimit T-DNA ends by initiating and terminating T-DNA transfer (Peralta and Ream *Proc. Natl. Acad. Sci.* **82:**5112-5116, 1985). Arrows show

10   direction and length of messenger RNAs (mRNAs) produced in gall tumors.

Figure 2 is a diagram of the auxin and cytokinin biosynthetic pathways encoded by the *A. tumefaciens* T-DNA oncogenes.

Figure 3 is a diagram of the binary plant transformation vector (pPEV6) (panel A) and of mutant transgenes that can be used to elicit co-suppression of T-

15   DNA oncogenes. "RB" and "LB" denote right and left T-DNA border sequences, respectively. NOS prom/NPTII denotes the nopaline synthase promoter fused to the neomycin phosphotransferase II gene (conferring kanamycin resistance). 5' UTR and 3' UTR denote the 5' and 3' untranslated regions, respectively, associated with the cauliflower mosaic virus promoter (CaMV 35S Pro). Arrowheads denote

20   respective restriction sites for *Xba*I, *Bam*HI, and *Eco*RI; numbers indicate respective coordinates relative to the vector sequence. Premature stop codons are shown in bold, whereas natural start codons are shown in plain text.

Figure 4 illustrates the most likely scenario for the creation of double-stranded RNA. The sense strand is transcribed from the CaMV 35S promoter and

25   the anti-sense strand is transcribed from the NOS promoter.

Figures 5(A)-5(F) depict several representative illustrations of bacterial-resistance constructs (BR construct) according to the invention. Promoters are designated by the capital letter "P" and the direction of expression is denoted by respective arrows. DNA sequences encoding untranslatable RNA molecules that

30   target specific genes are indicated; however, one of ordinary skill in the relevant art

will appreciate that untranslatable RNA molecules targeting other genes can be substituted. **Figure 5(A)** shows a BR construct in which one promoter (designated P_1) is used to express (direction of expression is indicated by the arrows) a DNA sequence encoding the sense-strand of a sequence that targets the *ipt* gene and the

5   sense-strand of a sequence targeting the *iaaM* gene. This same construct also contains a second promoter (designated P_2) that drives the expression of the antisense-strand of the *iaaM* gene and the antisense-strand of the *ipt* gene. Thus, the combination of both promoters will generate a double-stranded RNA that contains a section targeting the *ipt* gene and a section targeting the *iaaM* gene. **Figure 5(B)**

10   shows a BR construct that contains one promoter (P_1) that expresses a DNA sequence encoding several different untranslatable RNA sequences that target both the *ipt* gene and the *iaaM* gene. Starting at the 5' end of the construct, the promoter P_1 drives the expression of a sense-strand targeting the *ipt* gene, a sense-strand targeting the *iaaM* gene, an antisense-strand targeting the *iaaM* gene, and a sense-

15   strand targeting the *ipt* gene. This sequence can fold back on itself, and thus generate a double-stranded RNA molecule comprising regions homologous to both the *ipt* and *iaaM* gene. **Figure 5(C)** shows another variation of a BR construct. This construct comprises independent promoters, P_1 and P_2, to drive the expression of an untranslatable RNA molecule in both the sense and anti-sense orientations,

20   respectively. Two additional promoters (P_3 and P_4) are utilized for the expression of the sense and anti-sense expression of a second untranslatable RNA. **Figure 5(D)** shows another variation of the BR construct. This construct comprises one promoter (P_1) to express first the sense-strand and then the antisense-strand of an untranslatable RNA molecule targeting the *ipt* gene. This construct also comprises a

25   second promoter to express both the sense-strand and antisense-strand of an untranslatable RNA targeting the *iaaM* gene. **Figures 5(E)** and **5(F)** illustrate additional variations that can be used to express single-stranded untranslatable RNA molecules.

DETAILED DESCRIPTION

I.   Definitions and Abbreviations:

Untranslatable RNA molecule: An RNA molecule, either single-stranded or double-stranded, that is based on a naturally occurring RNA molecule but which,

5   as a result of genetic manipulation, is incapable of being translated to yield the full-length protein encoded by its naturally occurring counterpart. For example, such RNA can be rendered untranslatable through the introduction of one or more premature termination codons (TAA, TAG, or TGA), or by introduction of a mutation that shifts the reading frame. Alternatively, for example, the RNA may be

10   rendered untranslatable by the deletion of the AUG translation-initiation codon, or of the AAUAAA polyadenylation signal. The RNA may also be rendered untranslatable by simply removing a portion of the cDNA encoding the full-length protein. Thus, even if the sequence were translated, it would produce a truncated version of its naturally occurring counterpart.

15   **Antisense-strand RNA:** An RNA molecule that is complementary to a messenger RNA (which represents a corresponding "sense" strand).

          Disease-resistant transgenic plant: A "disease-resistant transgenic plant" as used herein is a transgenic plant exhibiting more resistance to gall disease than a corresponding non-transgenic plant.

20   **Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence whenever the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA

25   sequences are contiguous.

          Recombinant: A recombinant nucleic acid is one having a sequence that is not naturally occurring in the organism in which it is expressed, or has a sequence made by an artificial combination of two otherwise-separated, shorter sequences. This artificial combination is often accomplished by chemical synthesis or, more

30   commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g.,

by genetic engineering techniques. "Recombinant" is also used to describe nucleic acid molecules that have been artificially manipulated, but that contain the same control sequences and coding regions that are found in the organism from which the gene was isolated.

5   **Sense-strand RNA**: An RNA molecule that can serve directly as messenger RNA, i.e., that can be translated by ribosomes.

Double-stranded RNA: A "double-stranded RNA" is an RNA molecule that contains at least a 25-bp sequence including both a sense-strand and an antisense-strand. Double-stranded RNA molecules are additionally characterized by

10   the level of complementarity between the sense-strand and the antisense-strand. Complementarity refers to the amount of base pairing between the two sequences. For example, the sequence 5'-AUCGGAUAGU-3' is 100% complementary to the sequence 5'- ACUAUCCGAU-3' and 70% complementary to the sequence 5'-GAAAUCCGAU-3'. The double-stranded RNA should be of a sufficient length and

15   have a sufficient level of complementarity such that it confers disease resistance to the host cell (disease resistance can be determined using the assays described below). For example, the double-stranded RNA may have as little as 60% complementarity. Generally, however, double-stranded RNA sequences will have at least 70%, 75%, 80%, or 90% complementarity.

20   **Vector**: A "vector" is a nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences, such as an origin of replication, that permit the vector to replicate in a host cell. A vector may also include one or more screenable markers, selectable markers, or reporter genes and other genetic elements known in the art.

25   **Sequence identity**: The similarity between two nucleic acid sequences or between two amino acid sequences is expressed in terms of the level of sequence identity shared between the sequences. Sequence identity is typically expressed in terms of percentage identity; the higher the percentage, the more similar the two sequences.

Methods for aligning sequences for comparison purposes are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* **2**:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* **48**:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* **85**:2444-2448,

5   1988; Higgins and Sharp, *Gene* **73**:237-244, 1988; Higgins and Sharp, *CABIOS* **5**:151-153, 1989; Corpet et al., *Nucleic Acids Research* **16**:10881-10890, 1988; Huang, et al., *Computer Applications in the Biosciences* **8**:155-165, 1992; and Pearson et al., *Methods in Molecular Biology* **24**:307-331, 1994. Altschul et al., *J. Mol. Biol.*, **215**:403-410, 1990, presents a detailed consideration of sequence

10   alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST™, Altschul et al.. *J. Mol. Biol.*, **215**:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence-analysis programs blastp, blastn,

15   blastx, tblastn and tblastx. BLAST™ can be accessed at http://www.ncbi.nlm.nih.gov/BLAST/. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

For comparisons of amino acid sequences of greater than about 30 amino

20   acids, the "Blast 2 sequences" function in the BLAST™ program is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per-residue gap cost of 1). When aligning short peptides (fewer than about 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9,

25   extension gap 1 penalties). Proteins having even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 45%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity.

A first nucleic acid is "substantially similar" to a second nucleic acid if, when

30   optimally aligned (with appropriate nucleotide insertions or deletions) with the other

nucleic acid (or its complementary strand), nucleotide sequence identity occurs in at least about 60%, 75%, 80%, 85%, 90% or 95% of the nucleotide bases (As used herein, "optimally aligned" sequences exhibit a maximal possible sequence identity).

Sequence similarity can be determined by comparing the nucleotide sequences of

5   two nucleic acids using the BLAST™ sequence analysis software (blastn) available from The National Center for Biotechnology Information. Such comparisons may be made using the software set to default settings (expect = 10, filter = default, descriptions = 500 pairwise, alignments = 500, alignment view = standard, gap existence cost = 11, per residue existence = 1, per residue gap cost = 0.85).

10   Similarly, a first polypeptide is substantially similar to a second polypeptide if it shows sequence identity of at least about 75%-90% or greater when optimally aligned and compared using BLAST™ software (blastp) using default settings.

Bacterial resistance construct (BR construct): A "bacterial resistance construct" (abbreviated "BR construct") is a sequence of DNA that incorporates at

15   least one promoter and at least one DNA sequence encoding an untranslatable RNA (either double-stranded RNA, single-stranded RNA or a combination thereof). The arrangement of the promoter(s) in relationship to the DNA sequence(s) can vary. Representative configurations are illustrated in **Figures 5(A)-5(G). Figure 5(A)** shows a BR construct in which one promoter (designated P_1) is used to express

20   (direction of expression is indicated by the arrows) a DNA sequence encoding the sense-strand of a sequence that targets the *ipt* gene and the sense-strand of a sequence targeting the *iaaM* gene. This same construct also contains a second promoter (designated P_2) that drives the expression of the antisense-strand of the *iaaM* gene and the antisense-strand of the *ipt* gene. Thus, the combination of both

25   promoters will generate a double-stranded RNA that contains a section targeting the *ipt* gene and a section targeting the *iaaM* gene. **Figure 5(B)** shows a construct that contains one promoter (P_1) that expresses a DNA sequence that encodes several different untranslatable RNA sequences targeting both the *ipt* gene and the *iaaM* gene. Starting at the 5' end of the construct, the promoter P_1 drives the expression of

30   a sense-strand targeting the *ipt* gene, a sense-strand targeting the *iaaM* gene, an

antisense-strand targeting the *iaaM* gene, and a sense-strand targeting the *ipt* gene. This sequence can fold back on itself, and thus generate a double-stranded RNA molecule comprising regions homologous to both the *ipt* and *iaaM* gene. **Figure 5(C)** shows another variation of a BR construct. This construct comprises

5   independent promoters, P_1 and P_2, to drive the expression of an untranslatable RNA molecule in both the sense and anti-sense orientations, respectively. Two additional promoters (P_3 and P_4) are utilized for the expression of the sense and anti-sense strands of a second untranslatable RNA. **Figure 5(D)** shows another variation of the BR construct. This construct comprises one promoter (P_1) to express first the sense-

10   strand and then the antisense-strand of an untranslatable RNA molecule targeting the *ipt* gene. This construct also comprises a second promoter to express both the sense-strand and antisense-strand of an untranslatable RNA targeting the *iaaM* gene. **Figures 5(E)** and **5(F)** illustrate additional variations that can be used to express single-stranded untranslatable RNA molecules. **Figures 5(A)-(F)** are intended to

15   illustrate a few of various different promoter/DNA sequence combinations that are possible. A person of ordinary skill in the art will appreciate that many additional combinations can be used in BR constructs according to the invention.

II.   General Description

20   The present method provides new and effective methods for suppressing the expression of *Agrobacterium* oncogenes. In particular, this invention is directed at, *inter alia*, producing plants that are capable of substantially inhibiting the formation of bacterially induced galls. These plants are produced by selecting a target gene of *Agrobacterium* origin, designing a nucelic acid (typcially DNA) BR construct

25   encoding untranslatable single-stranded RNA, double-stranded RNA, and/or untranslatable double-stranded RNA molecules having a high sequence identity to the target gene, and introducing at least one of these BR constructs into a host cell.

a.   Selection of the Target Gene

There are many species of *Agrobacterium*, for example *Agrobacterium tumefaciens, Agrobacterium vitis, Agrobacterium rubi,* and *Agrobacterium rhizogenes*. The nucleic acid sequences of the respective oncogenes contained

5   within these species are highly conserved. Therefore, a BR construct designed to encode at least one RNA molecule having high sequence identity to one or more target tumor-inducing *Agrobacterium* genes will likely confer resistance to at least one (typically multiple) species of *Agrobacterium*.

10   ### b.   Producing a Nucleic Acid Construct Encoding an Untranslatable RNA

Practicing the present invention requires the manipulation of a DNA sequence using molecular biological techniques to produce the desired nucleic acid construct. The DNA sequence can be manipulated using standard procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by

15   exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate or with the use of specific oligonucleotides in combination with PCR. Details of these techniques are provided in standard laboratory manuals such as Sambrook et al., *Molecular Cloning: A Laboratory*

20   *Manual,* 2nd ed., Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989, and Ausubel et al., *Short Protocols in Molecular Biology,* 2nd ed., John Wiley and Sons, 1992.

Suppression of an oncogene using a method according to this invention is achieved by expressing, in a host cell, molecules of a BR construct having high

25   sequence identity to the target gene(s). At least one molecule of the BR construct having substantial sequence identity to the target gene(s) is introduced into a host cell. The BR construct can be delivered into the host cell genome by delivering into the host a vector cell containing the BR construct.

Application of the present invention to construction of a BR construct

30   desirably involves the selection of appropriate control sequences such as promoters,

enhancers, and 3'-untranslated regions. The choice of which type of control

sequence(s) to use in a BR construct will depend upon the particular tissue in which

the BR construct will be expressed and the particular sequence(s) of the target

gene(s). The variety of plant that is selected for transformation will also affect the

5   choice of the control sequence(s), the vector, and the transformation method.

Constructing a suitable BR construct involves the selection of a DNA

sequence that will be used to target at least one specific bacterial gene. Generally,

the DNA sequence will encode an untranslatable RNA having at least 70% sequence

identity with the target bacterial gene(s) (i.e., the gene(s) that will be suppressed).

10   In producing the BR construct, a DNA molecule homologous to a target

bacterial gene can be manipulated to render a single-stranded RNA or double-

stranded RNA molecule encoded by the DNA molecule untranslatable. This can be

done by, e.g., introducing any of various stop signals into the open reading frame of

the DNA molecule to produce the BR construct. For example, the BR construct can

15   be configured to contain one or more premature stop codons. A "stop codon" is a

codon of the genetic code that signals ribosomes to terminate translation of a

messenger RNA molecule. There are three stop codons in the genetic code: TAA,

TAG, and TGA, as illustrated in Table 1 below.

TABLE 1

The Genetic Code

First Position	Second position				Third Position
(5' end)	T	C	A	G	(3' end)
T	Phe	Ser	Tyr	Cys	T
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP (och)	STOP	A
	Leu	Ser	STOP (amb)	Trp	G
C	Leu	Pro	His	Arg	T
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	T
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	T
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val (Met)	Ala	Glu	Gly	G

In the context of the foregoing discussion, a "premature" stop codon is located at a position transcriptionally upstream of a naturally occurring stop codon. Generally, the premature stop codon is positioned within 100 nucleotides of the translation-start codon, and typically within 50 nucleotides of the translation-start codon.

One or more stop codons can be inserted into the DNA sequence to produce the BR construct. Alternatively, the DNA sequence can be mutated to convert an

existing triplet codon (encoding an amino acid) of the sequence into a stop codon.

For example, the hypothetical DNA sequence:

... CTT AAC TGG ·TCC ...

5

would normally produce a corresponding RNA translated by ribosomes to produce

the polypeptide:

... Leu Asn Trp Ser ...

10

The corresponding RNA can be rendered untranslatable by introducing a stop codon,

such as TAA, into the DNA sequence. For example, the modified DNA sequence:

... CTT TAA AAC TGG TCC ...

15

encodes:

... Leu STOP

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20   Alternatively, in the foregoing hypothetical DNA sequence, substitution of the ninth

nucleotide (guanosine (G)) by adenosine (A) will result in the creation of a

premature stop codon, rendering the corresponding RNA untranslatable, i.e., the

following DNA sequence is produced:

... CTT AAC TGA TCC ...

25

which encodes:

... Leu Asn STOP

30

        Alternatively, the addition or deletion of one or more bases into or from,

respectively, the DNA sequence (except multiples of three bases) can be used to

displace the reading frame of the DNA sequence which can render the corresponding

RNA molecule untranslatable. For example, the introduction of a single nucleotide

(G) into the first codon of the foregoing hypothetical DNA sequence creates a new reading frame containing a premature stop codon:

... CTG TAA CTG GTC C ...

5   which encodes:

... Leu STOP

10   An alternative manner of rendering an RNA molecule untranslatable is to remove the translation-initiation codon (usually ATG) from the corresponding DNA sequence. However, this method may result in use by ribosomes of an ATG codon elsewhere in the DNA sequence to initiate translation. Removal of the initiation codon ATG is, therefore, desirably used in conjunction with inserting one or more
15 premature stop codons into a DNA sequence.

          Another method of introducing a premature stop codon is by truncating the subject DNA sequence. This can be done, for example, by using a restriction enzyme to excise a portion of a target gene and then cloning only the excised portion of the gene into a vector. Transcription of the vector will produce a segment of
20 homologous RNA effective to produce a co-suppression phenomenon, but will not produce a fully processed protein. Currently, the mechanism by which co-suppression works is not known. However, the phenomenon has been previously described in detail (Lindbo and Dougherty, *Virology* **189**:725-733, 1992).

          One or more of the above approaches may be used to render an RNA
25 molecule encoded by a BR construct untranslatable. It will be appreciated that a very large number of sequence changes can be made in a suitable manner, the precise nature of which may depend on the actual DNA sequence being manipulated, to render the sequence of the corresponding RNA untranslatable. In certain embodiments, more than one stop codon may be introduced into the DNA sequence.
30 For example, the resulting BR construct may contain a DNA sequence encoding untranslatable RNA that has been altered to contain at least one stop codon situated early in the open reading frame; the open reading frame is thus "truncated."

Many standard DNA mutagenesis techniques are available to modify DNA sequences in a manner as described above to produce the BR construct. The mutagensis techniques include, for example, M13 primer mutagenesis. Details of such techniques are provided in Sambrook et al., Chapter 15, *Molecular Cloning: A*

5   *Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1989; and in Ausubel et al., Chapter 8, *Short Protocols in Molecular Biology,* 2nd ed., John Wiley and Sons, 1992.

According to another aspect of the invention, double-stranded RNA molecules are provided that are homologous to a target bacterial gene. Each strand

10   of the double-stranded RNA can be produced from separate BR constructs, wherein a first BR construct encodes the antisense-strand and a second BR construct encodes the sense-strand. Upon transcription of the first and second BR constructs, the resulting sense-strand and corresponding antisense-strand form double-stranded RNA.

15   Alternatively, the sense-strand and antisense-strand can be produced from a single BR construct. The single BR construct can utilize two distinct promoters to initiate the expression of the antisense-strand and the sense-strand, or one promoter to drive expression of both strands. When a single promoter is utilized, both the sense-strand and the antisense-strand are normally produced as a single transcript,

20   wherein the RNA sequence will fold back on itself to create a double-stranded RNA. Similarly, multiple combinations of promoters, antisense-strands, and sense-strands can be encoded on the same BR construct.

Double-stranded RNA can also be produced by transforming cells with a BR construct that encodes the sense-strand, a self-cleaving ribozyme, and the antisense-

25   strand all on a single transcript. Upon transcription of such a BR construct, the sense-strand, the self-cleaving ribozyme, and the antisense-strand are produced. The self-cleaving ribozyme sequence folds back on the resulting transcript and splices the transcript between the sense- and antisense-strands, generating two independent complementary strands of RNA.

A proposed mechanism for suppressing a target bacterial gene by introducing into a host cell molecules of a BR construct having one or more sequences homologous to at least a portion of the target gene typically involves the activity of an inducible, cellular activity that degrades specific RNA sequences. This

5   mechanism requires that the untranslatable single-stranded RNA, double-stranded RNA, and/or untranslatable double-stranded RNA encoded by the BR construct be recognizable by the RNA-specific cellular activity. To such end, the target DNA sequence and the DNA sequence of the BR construct desirably share a sequence identity.

10   In the Examples presented below, the DNA sequences of the respective BR constructs are derived directly from corresponding target bacterial nucleotide sequences. The sequences of the respective BR constructs differ from the corresponding target sequences primarily only by the presence (in the sequences of the BR constructs) of premature stop codons inserted by mutagenesis involving one

15   or more frame-shift mutations. Thus, in these examples, the BR constructs have over 90% sequence identity to the corresponding target DNA sequences. However, a BR construct needs only to have sufficient sequence identity to the target gene so that the BR construct confers disease resistance to the host cell. Therefore, a BR construct that encodes an RNA molecule having less than 90% sequence identity to

20   the corresponding target DNA sequence can be used. In any event, the BR construct typically has at least 60% sequence identity to the corresponding target DNA molecule, and in some cases at least 70%, 75%, or 80% sequence identity to the target DNA molecule.

The following examples also describe BR constructs in which the DNA

25   sequence corresponds to 700 base pairs of the ORF (open reading frame) of the target gene. It will be understood, however, that the untranslatable RNA provided by the BR construct need not correspond specifically to 700 base pairs of the ORF of the target gene; substantially shorter sequences of untranslatable RNA may also be effective.

The BR construct should be sufficiently long and share a sufficient level of
sequence identity to the target gene such that the BR construct confers disease
resistance to the host cell. The ability of a particular BR construct to confer disease
resistance to a host cell can readily be determined by using the assay described

5   below, in which a transgenic plant is challenged with a bacterial pathogen.
Typically, the ORF of the DNA sequence of the BR construct is at least 25-100
nucleotides in length, although longer sequences, such as 100-250, 250-500, or
greater than 500 nucleotides may be used and may provide superior results. In
embodiments where shorter sequences (i.e., less than 250 nucleotides) are utilized,

10   the respective BR constructs will likely share a high degree of sequence identity
(e.g., at least 60% sequence identity) with the target gene sequence.

The present invention can be used to suppress the expression of a bacterial
tumor gene in a plant cell. To such end, the plant cell is transformed with a BR
construct having a high sequence identity to a target bacterial gene. The molecule of

15   the BR construct encodes one or more untranslatable single-stranded RNA and/or
double-stranded RNA molecules. The BR construct can comprise a sequence of at
least 25 nucleotides in length having at least 60% sequence identity to the target
bacterial gene. Alternatively, the BR construct can comprise a sequence of at least
100 nucleotides in length having at least 60% sequence identity to the target

20   bacterial gene. Furthermore, the BR construct can comprise a nucleic acid sequence
of at least 250 nucleotides in length having at least 60% sequence identity to the
target bacterial gene.

The present invention is not limited to the suppression of a single target
bacterial gene. Rather, it is possible to suppress, according to the invention, the

25   expression of multiple target genes using one or more BR constructs that comprise
DNA sequences corresponding to several respective genes expressed in the target
cell. This approach can be used to enhance resistance to a single bacterial strain or
to produce resistance to multiple strains of bacteria in a single plant. As illustrated
in the Examples below, the formation of a gall on a plant by a pathogenic bacterium

30   infecting the plant can be reduced or eliminated by the introduction into the cells of

the plant, a BR construct having a high sequence identity to at least one gene of the

infecting bacterium. This approach allows the production of transgenic plants

resistant to the bacterium.

5   c.   **Vectors**

It will be appreciated that the efficacy of the present invention depends on

transcription of the DNA sequence of the BR construct introduced into a host plant

cell. Transcription of a DNA sequence into messenger RNA is preferably effected

by including a promoter region at the 5' end of the DNA sequence of the BR

10   construct, together with regulatory sequences at the 3' end of the DNA sequence.

Many promoter sequences and 3'-regulatory sequences have been identified and

characterized in a wide range of viral, bacterial, fungal, plant, and animal genes.

Therefore, the selection of suitable promoter and a suitable 3'-regulatory sequence is

within the capability of a person of ordinary skill in the relevant art.

15   After choosing and incorporating the appropriate regulatory DNA

sequence(s) into the BR construct, the BR construct is incorporated into a suitable

vector for introduction into the host cell. The choice of regulatory sequences, as

well as the choice of a suitable transformation vector will depend upon the type of

host cell into which the BR construct is to be introduced and upon the desired

20   transformation method for the host cell. Vectors suitable for transforming plant cells

(derived from either monocotyledonous or dicotyledonous plants) are described

below. Transformation of both monocotyledonous and dicotyledonous plant cells is

now routine and the selection of the most appropriate transformation technique will

be determined by the skilled practitioner.

25   A number of promoters that are active in plant cells have been described in

the literature. Promoters that are known to cause transcription of DNA in plant cells

can be used to suppress gene expression in plants. Such promoters can be obtained

from plant cells or from plant viruses and include, but are not limited to, the CaMV

(cauliflower mosaic virus) 35S promoter. It is desirable that the chosen promoter be

30   capable of causing sufficient expression of the BR construct to produce sufficient

RNA to suppress expression of the target gene. The amount of RNA needed to

suppress expression of a particular target gene may vary from one target gene to

another, expression level of the particular target gene, and the plant type.

Accordingly, whereas the CaMV promoter is employed in the Examples presented

5   below, it will be understood that this promoter may not be optimal for all

embodiments of the present invention.

The vector can be configured to optimally exploit the promoter and

termination sequences in the target gene itself. Used in this way, the target-gene

promoter is not altered and the subsequent stop signals are introduced into the ORF

10   of the target gene. Additionally, the termination signals of the target gene can be left

intact. This configuration permits increasing the transcription of untranslatable

RNA and/or double-stranded RNA whenever the factors necessary for transcription

of the target gene are present.

The promoter(s) used in a BR construct according to the invention may be

15   modified, if desired, to include additional regulatory sequence(s). Such additional

regulatory sequences can confer, e.g., specificity to the respective promoter region.

For example, the small subunit of the ribulose bis-phosphate carboxylase ("ss

RUBISCO") gene is expressed in plant leaves but not in root tissues. A sequence

motif that represses the expression of the ss RUBISCO gene in the absence of light

20   (to create a promoter which is active in leaves but not in root tissue), has been

identified. This and/or other regulatory sequences such as the HSP70 leader

sequence from petunia, may be ligated to promoters such as the CaMV 35S, or

figwort mosaic virus (FMV) promoter, to modify the expression patterns of a gene.

The 3'-nontranslated region (that functions as a polyadenylation site for RNA

25   in plant cells) of a gene can also be used in the present invention. Such 3'-non-

translated regions include, but are not limited to, the 3'-transcribed (but non-

translated) region of the CaMV 35S gene, the pea-rbcS E9 3'-non-translated region,

the NOS 3'-non-translated region, and one or more of the 3'-transcribed, non-

translated (but non-translated) regions containing the polyadenylation signals of the

tumor-inducing (Ti) genes of *Agrobacterium*, such as the tumor morphology large (*tml*) gene.

d.   Plant Species

5   Gall formation in plants can be induced by any of various organisms. For example, galls can be induced by bacteria from the genus *Agrobacterium*, and certain species of *Pseudomonas, Rhizobacter*, and *Rhodococcus.*

In the case of *Agrobacterium*, gall formation is caused by genes introduced into the plant cells on the Ti plasmid provided by an infecting bacterium.

10   There are a variety of naturally occurring Ti plasmids that can give rise to different types of galls. Galls are normally caused by *A. tumefaciens* which infects over 391 genera of plants including shrubs, fruit trees, nut trees, tuber-producing plants, conifers, grape vines, chrysanthemums, roses, cane berries, nursery crops, and commercially important plants (Bradbury, J.F. Guide to Pathogenic Bacteria. CAB

15   International, Slough, Britain, 1986). Hairy root is another type of gall induced by *A. rhizogenes*, that primarily affects the roots of apple trees. Finally, *Agrobacterium rubi* can infect both raspberries and black berries, and give rise to cane galls. (Agrios, *Plant Pathology,* Academic Press, 1997).

20 ### e.   Transformation

A BR construct according to the present invention is introduced into a plant cell using a suitable vector and transformation method as described below. The resulting transformed cells can then be allowed to regenerate into whole plants. Any of various conventional methods employed for transforming plant cells to generate

25   transgenic plants, including stably transformed plants, can be used in this invention. The choice of method will typically vary with the type of plant to be transformed; persons skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome mediated transformation; polyethylene

30   glycol-mediated transformation; transformation using viruses; microinjection of

plant cells; microprojectile bombardment of plant cells and *Agrobacterium tumefaciens* (AT)-mediated transformation. The DNA sequences comprising the CaMV 35S promoter and CaMV 35S non-translated 3' region and the mutated cDNA encoding a untranslatable RNA can be combined in a single cloning vector.

5 This vector can be transformed subsequently into, e.g., cultured tobacco cells.

f.   Conferred Resistance

The mechanism of co-suppression is unknown. However, transgenic tissue containing a co-suppression gene can be grafted onto a non-transgenic plant. The

10 graft then confers resistance to the non-transgenic plant (Jorgensen et al., *Science*, **279**:1486-87, 1998). Furthermore, this "conferred resistance" can be established by either grafting a scion (upper vegetative tissue) onto a transgenic stock (lower vegetative tissue), or by grafting a transgenic scion onto a non-transgenic stock. This conferred resistance can be transmitted through at least 30 cm of non-transgenic

15 tissue.

It is possible that the phenomenon of conferred resistance may allow resistance to gall formation to be introduced into existing plants. Thus, a mature plant can be made resistant to gall formation by grafting transgenic tissue onto the plant. Furthermore, several grafts can be placed independently on the plant to

20 produce resistance in the plant to several different bacterial pathogens.

A grafting protocol can be made more flexible by utilizing cross-species grafting. Thus, a graft from, e.g., almond (*Prunus amygdalus*) can be placed on stock from peach (*Prunus persica*). Such flexibility increases the range of plants for which a given transgenic tissue can be used. For example, after almond tissue

25 containing the desired BR construct has been generated, the tissue can be used to confer resistance not only to other almond trees, but also to peach trees.

The use of conferred resistance also decreases the possibility that a transgene made according to the invention will be introduced into the environment. This is because the resistance is derived from the graft itself and the rest of the plant

remains free of the transgene. Therefore, the fruits and seeds of the plant can be left non-transgenic, so as not to transmit the gene.

By preventing a transgene from being systemically introduced into the plant, products for human consumption can be produced by the plant that are not

5   transgenic.

III.   EXAMPLES

a. Construction of BR Constructs Encoding Untranslatable RNA molecules

10   To produce a DNA construct capable of eliciting co-suppression, the *iaaM* and *ipt* oncogenes were modified by changing the third codon of each gene to a stop codon and introducing a frameshift mutation downstream of each respective introduced stop codon, thereby creating additional stop codons in the reading frame (**Figure 3**). The polymerase chain reaction (PCR) primer oligonucleotides used to

15   create these mutations in the DNA construct are described in Table 2. The primers used to modify the *ipt* gene resulted in the deletion of the 5' C of the third codon and changed the 3' G of the third codon to an A. The *iaaM* modifications introduced the stop sequence TGA at the third codon and the two bases following the third codon were deleted. A second DNA construct was made that contained a hybrid transgene

20   configured to elicit co-suppression of both *ipt* and *iaaM*. The second DNA construct was made by digesting a vector (containing the *ipt*-stop mutation) and a vector containing the *iaaM*-stop mutation with *Bam*H1. The fragment containing the *iaaM*-stop mutation was then ligated into the fragment containing the *ipt*-stop mutation, thereby yielding a BR construct containing the mutant *iaaM* gene inserted into the

25   mutant *ipt* gene (**Figure 3**). The sequence of each DNA construct was verified, and the BR construct was inserted into a plant-transformation vector (pPEV6; Lindbo and Dougherty, *Mol. Plant-Microbe Interact.* **5**:144-153, 1992) oriented such that the sense-strand of each mutant was expressed from the cauliflower mosaic virus (CaMV 35S) promoter (**Figure 3**).

30

Table 2

PCR Primers Used to Produce Mutant *iaaM* and *ipt* Genes

Primers		Sequence
iaaM stop	5' primer	5'-CGGGATCCATGTCATGAACCTCTCCTTGA TAAC-3' (SEQ ID NO: 1)
	3' primer	5'-CGGGATCCTGCGACTCATAGT-3' (SEQ ID NO: 2)
ipt stop	5' primer	5'-GAAGATCTGATCATGGACTGAATCTAAT TTTCGGTCC-3' (SEQ ID NO: 3)
	3' primer	5'-GAAGATCTGATCACTAATACATTCCGAAC GG-3' (SEQ ID NO: 4)

b.   Construction of Double-Stranded RNA and Untranslatable Double-Stranded RNA Vectors

A vector encoding an *iaaM*-stop mutation (i.e., a DNA construct modified to incorporate a stop codon at the third codon, followed by a two base pair deletion) exhibited evidence of bacterial resistance. This resistance was derived from the production of double-stranded RNA by transcription of the vector (**Figure 4**). The sense-strand of the double-stranded RNA was transcribed from the CaMV 35S promoter and the antisense-strand was probably transcribed from the nopaline synthase promoter (NOS). Thus, this vector can produce a long double-stranded RNA that is approximately 2,000 bp long. Evidence of such a double-stranded RNA was found in northern blots that showed partially degraded antisense-strands ranging in size from 3.4 Kb to less than 0.24 Kb. As is depicted in Figure 4, the double-stranded RNA appeared to include an approximately 2,000 bp long region that was 100% homologous to the *iaaM* sequence. Therefore, bacterial resistance observed in *iaaM*-transgenic plants is probably attributable to the presence of the double-

stranded RNA. This resistance does not appear to have been due solely to the presence of the introduced stop codon.

Double-stranded RNA is capable of suppressing endogenous plant genes, as well as suppressing viral infection (Ruiz et al., *Plant Cell* **10**:937-946, 1998).

5   However, the suppression, via double-stranded RNA and/or untranslatable RNA, of disease symptoms caused by bacteria, such as *Agrobacterium,* has previously not been shown.

c.   **Production of Plants Homozygous for Untranslatable RNA Transgenes**

10   Mutant *iaaM* (SEQ ID NO: 7), *ipt* (SEQ ID NO: 5), and *ipt/iaaM* genes (SEQ ID NO: 9) were introduced separately into haploid tobacco (*N. tabacum,* "Burley" and "Kentucky" cultivars). The chromosomes of kanamycin-resistant transformants of these plants were doubled to produce plants homozygous for each transgene. These plants were the subjects of initial tests, which were repeated on the

15   F1 progeny of self pollinations.

d.   **Gall-resistant Plant Phenotypes**

Putative *iaaM*-suppressing plants (designated "TDP1" lines) were challenged with *ipt*-mutant *A. tumefaciens* (strain 338; Garfinkel et al., *Cell* **27**:143-153, 1981).

20   Of 63 lines tested, 35 lines, including TDP1-B7 & TDP1-B17, suppressed the wild-type *iaaM* gene on *A. tumefaciens* T-DNA introduced into such plants, thereby preventing tumorous growth in the respective plants. When inoculated with wild-type *A. tumefaciens* (strain A348; Garfinkel et al., *Cell* **27**:143-153, 1981), each of the 35 lines produced "shooty" galls typical of cytokinin-driven tumors, such as

25   those induced on wild-type plants inoculated with *iaaM*-mutant *A. tumefaciens* (Ream et al., *Proc. Natl. Acad. Sci. USA* **80**:1660-1664, 1983; and Garfinkel et al., *Cell* **27**:143-153, 1981). Thus, the plants that produced shooty galls exhibited the phenotype expected of a transgenic plant capable of suppressing *iaaM*.

Putative *ipt*-suppressing plants (designated "CW1" lines) were challenged

30   with *iaaM*-mutant *A. tumefaciens* (strain 328; Garfinkel et al., *Cell* **27**:143-153,

1981). Of 45 lines investigated, one (designated "CW1-K27") clearly suppressed the wild-type *ipt* gene on introduced *A. tumefaciens* T-DNA. Another 6 lines exhibited similar results in initial tests on the original transgenic plants, but these lines were lost before seeds were obtained. The *ipt*-suppressing plant (CW1-K27),

5   when inoculated with wild-type *A. tumefaciens*, produced small necrotic galls typical of auxin-driven tumors, such as those induced on wild-type plants inoculated with *ipt*-mutant *A. tumefaciens* (Ream et al., *Proc. Natl. Acad. Sci. USA* **80**:1660-1664, 1983; Garfinkel et al., *Cell* **27**:143-153, 1981). There were 30 lines tested (designated "CW4" lines) that contained the hybrid *ipt-iaaM* transgene. One line

10   (designated "CW4-B30") exhibited a significant reduction in tumorigenesis when inoculated with either wild-type, *iaaM*-mutant, or *ipt*-mutant *A. tumefaciens*. Although the CW4-B30 line exhibited markedly reduced gall size and absence of gall formation at some inoculation sites, gall tumorigenesis was not entirely absent. The controls were kanamycin-resistant plant lines (designated "PEV6" lines) that

15   contained the transformation vector T-DNA (from pPEV6) without a transgene located downstream of the CaMV 35S promoter. As expected, none of these lines exhibited any suppression of T-DNA oncogenes upon infection with *A. tumefaciens*.

e.   Molecular Analysis of Transgenic Plants

20   To examine transgene copy number and structural integrity, Southern blot analyses were performed on representative plant lines of each type. Most lines contained several copies of the transgene, and all lines contained one or more intact copies (see Tables 3, 4, and 5, below). Some lines also contained rearranged copies of the transgene, which were identified by the presence of multiple bands in the

25   Southern blots.

TABLE 3
Southern Blot of Genomic DNA Digested with *Bam*H1

Transgenic Plant	1799-bp Transgene Fragment (+/-)*	Oncogene Suppression (+/-)*
CW4 B1 (ipt-stop/iaaM-stop)	+	-
CW4 B11 (ipt-stop/iaaM-stop)	+	-
CW4 B22 (ipt-stop/iaaM-stop)	+	-
CW4 B30 (ipt-stop/iaaM-stop)	+	+/-
TDP1 B7 (iaaM-stop)	+	+
TDP1 B17 (iaaM-stop)	+	+
TDP1 B27 (iaaM-stop)	+	+
TDP1 B31 (iaaM-stop)	+	-
PEV6 B2 (vector alone)	-	-
PEV6 B14 (vector alone)	-	-
* + denotes the presence of the transgene fragment or the suppression of oncogene expression. - denotes the absence of the transgene fragment or the absence of oncogene expression.		

5

TABLE 4
Southern Blot of Genomic DNA Digested with *Eco*R1

Transgenic Plant	Transgene Copy Number	Oncogene Suppression (+/-)*
TDP1 B7 (iaaM-stop)	3-4	+
CW4 B1 (ipt-stop/iaaM-stop)	2	-
CW4 B11 (ipt-stop/iaaM-stop)	2	-
CW4 B22 (ipt-stop/iaaM-stop)	2	-
CW4 B30 (ipt-stop/iaaM-stop)	4	+/-
TDP1 B17 (iaaM-stop)	2	+
TDP1 B27 (iaaM-stop)	1	+
TDP1 B31 (iaaM-stop)	4<	-
PEV6 B2 (vector alone)	0	-
PEV6 B14 (vector alone)	0	-
* + denotes the presence of the transgene fragment or the suppression of oncogene expression. - denotes the absence of the transgene fragment or the absence of oncogene expression.		

10

TABLE 5
Southern Blot of Genomic DNA Digested with
either *Hin*f1 or *Eco*R1 Digested Genomic DNA

Transgenic Plant	Presence of Restriction Fragment (+/-)*	Restriction Enzyme	Suppression of Oncogenes (+/-)*
CW1 K27 (ipt-stop)	+	*Hin*f1	+
CW1 K27 (ipt-stop)	+	*Eco*R1	+
CW1 K52 (ipt-stop)	+	*Hin*f1	-
CW1 K52 (ipt-stop)	+	*Eco*R1	-
* + denotes the presence of the transgene fragment or the suppression of oncogene expression. - denotes the absence of the transgene fragment or the absence of oncogene expression.			

The foregoing results show that co-suppression drastically reduces accumulation of mRNA encoded by the eliciting transgene and mRNA encoded by other genes that contain sufficient (approximately 70% or more) sequence similarity (Marano and Baulcombe, *Plant J.* **13:**537-546, 1998). Hence, Northern blot analysis was used to measure mRNA accumulation (but not transcription rate). In all *iaaM*-suppressing lines that were examined, transgene mRNA was undetectable, as expected, using a strand-specific hybridization probe. However, full-length and degraded antisense RNA molecules that included the transgene sequences were detected using a double-stranded hybridization probe. These transcripts appear to have initiated from the vector's nopaline synthase promoter and continued through both the neomycin phosphotransferase gene (in the sense direction) and the *iaaM* transgene (in the antisense direction). In the successful *ipt*-suppressing line, a trace amount of transgene-encoded mRNA was detected. Another line that contained the *ipt* transgene accumulated a large amount of transgene mRNA, but this line failed to co-suppress an incoming *A. tumefaciens* T-DNA-borne *ipt* gene. Plants that contained the hybrid *ipt-iaaM* transgene accumulated varying quantities of transgene-encoded mRNA. As expected, most of these lines did not elicit any co-suppression and only one line elicited partial co-suppression.

f.   Discussion

By studying the ability of wild-type and *iaaM-* or *ipt*-mutant *A. tumefaciens* to induce tumors on various lines of transgenic plants, it has been shown that: 1) many lines suppressed an incoming T-DNA *iaaM* gene, 2) one line suppressed an incoming T-DNA *ipt* gene, and 3) one line partially suppressed both *ipt* and *iaaM* genes simultaneously.

By combining successful *iaaH-* or *iaaM*-suppressing transgenes with an *ipt*-suppressing transgene in a single plant line, a transgenic plant fully resistant to gall formation can be produced. This can be done through standard breeding techniques, in which a cross between lines homozygous for the individual genes is performed. The F2 generation will include plants homozygous for both transgenes; such plants are expected to substantially resistant to gall formation. This expectation is supported by data from a plant that contained the hybrid *ipt-iaaM* transgene. This plant nearly prevented gall formation, indicating that a single hybrid gene can elicit co-suppression against two target oncogenes.

The transgenes used in this study will likely elicit co-suppression against a broad variety of *Agrobacterium* species, as well as various strains of such species. This allows the production of plants (such as grape vines, fruit trees, nut trees, and chrysanthemums) that are resistant to gall formation. Other studies have established that approximately 70% sequence identity is required to elicit co-suppression (Marano and Baulcombe, *Plant J.* **13**:537-546, 1998). Fortunately, the target T-DNA oncogenes (*iaaM* and *ipt*) are highly conserved among diverse *A. tumefaciens* strains. Even limited-host-range grape-specific *A. vitis* strains have greater than 90% sequence identity with the octopine-type oncogenes used in our study (Otten and DeRuffray, *Mol. Gen. Genet.* **245**:493-505, 1994).

Having illustrated and described the principles of the invention in multiple embodiments and examples, it should be apparent to those skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. Therefore, the invention encompasses all modifications coming within the spirit and scope of the following claims.